Detailed Material and Methods

**Classical murine model of parabiosis.** Parabiotic mouse pairs were created to investigate the mobilization and incorporation of progenitors to vessel formation in normal and ischemic conditions using a hindlimb ischemia model of neovascularization (Fig. 1a; upper panel). Parabiotic partners share all major histocompatibility antigens and, thus, are free of immunological barriers to cell migration and neovascularization. Weissman and colleagues have previously shown that parabiosis of syngenic males to females does not result in a detectable anti H-Y immune response \(^1\). Unambiguous cell tracking between the mice was possible by assaying for genetic markers unique to one animal in the pair. We surgically joined transgenic animals constitutively expressing β-galactosidase (β-gal) under transcriptional regulation of the Tie2 promoter (LacZ\(^-\); Jackson Laboratory, Bar Harbor, Maine) \(^2\), and wild type animals (LacZ\(^-\)), both of the FVB/N strain. The Tie2 receptor is expressed in endothelial lineage cells that participate in neovascularization \(^2\). Briefly, mice were anesthetized to full muscle relaxation with xylazine and ketamine HCl (1.67 mg per 10 g of body weight) by intraperitoneal injection and joined by a modification of the technique of Bunster and Meyer \(^3\). After shaving the corresponding lateral aspects of each mouse, matching skin incisions were made from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create about ½ cm of free skin. The olecranon and knee joints were attached by a single 2-0 silk suture and tie, and the dorsal and ventral skins were approximated by staples \(^1,4\). Cross-circulation in these animals was confirmed by tracking the flow of Evans blue dye from mouse A to mouse B following intravenous injection. Consecutive peripheral blood chimerism of parabiotic mice was determined in preliminary tests using CD45 allotype analysis \(^5\). Following the surgical procedure, blood was drawn after an increasing time interval from the parabiotic partners that differ in their CD45 locus (CD45.1 and CD45.2; Jackson Laboratory). The samples were stained for 30 min at 4°C with anti-CD45.1-FITC and anti-CD45.2-PE (both ebiosciences, San Diego, CA), respectively, lysed with hypotonic FACS lysing solution (BD biosciences) for 5 min, and washed twice with PBS prior to analysis on a FACSCalibur (BD biosciences). For the final
experiments, hindlimb ischemia was induced in the wild type animal 4 weeks after generation of the parabiotic pairs.

**Reverse bone marrow transplantation model.** To investigate the source of TPC, we conducted experiments with a modified model of mouse parabiosis ([Fig. 1a; lower panel](#)). In those experiments, LacZ⁺ mice were transplanted with bone marrow from LacZ⁻ mice (identifying TPC derived from other sources than the bone marrow) prior to the generation of parabiotic pairs. For this purpose, recipient mice were lethally irradiated with 9.5 Gy and received intravenous injection of 2 x 10⁶ donor bone marrow cells each. To determine the transplant efficiency of our protocol, we transplanted bone marrow from CD45.1 mice into CD45.2 mice revealing a transplantation efficacy of 95.2±1.7% after 6 weeks. Therefore, for the final experiments, transplanted LacZ⁺ animals were used 6 weeks following the reverse bone marrow transplantation to generate the respective parabiotic pairs.

**Rat model of heterotopic small intestine transplantation.** We used a rat model of heterotopic small intestine transplantation as previously described. To harvest the donor small intestine, the colon arteries, the portal vein and the pylorus vein were ligated followed by ligation of the caval vein. After ligation of the gastric and splenic veins, the pancreas was separated from the small intestine. Then, the aorta was dissected free below the mesenteric artery and ligated. Then the small intestine was separated from the caecum, the aorta was perfused with cold sodium chloride solution to remove all blood cells from the donor organ, and the small intestine was washed with cold sodium chloride solution. The donor small intestine was transferred to the recipient rat and the aorta of the donor organ was connected by end-to-side anastomosis to the aorta of the recipient whereas the portal vein of the donor organ was connected by end-to-side anastomosis to the caval vein of the recipient. When appropriate perfusion of the transplanted organ was established, the distal part of the donor small intestine and the caecum of the recipient were joined by end-to-side anastomosis whereas the proximal part of the small intestine was ligated. Hindlimb ischemia was induced three weeks after organ transplantation.

**Rat model of orthotopic liver transplantation.** We used a rat model of physiological liver transplantation as previously described. Transplantations were performed either sex-mismatched in
Lewis rats or in an allogeneic fashion using DA rats as donors and PVG rats as recipients (both from Harlan-Winkelmann, Borchen, Germany). The latter model is characterized by spontaneous tolerance. To harvest the donor liver, the caval vein was sub-diaphragmatically ligated and the gastric vein including side branches were also ligated. Then the portal vein was dissected free and the hepatic artery was ligated. After ligation of the distal caval vein (above the suprarenal vein) and the portal vein, the common biliary duct was prepared using the Cuff technique and the liver was perfused using cold sodium chloride solution. In a similar procedure, the liver of the recipient rat was removed and discarded. For implantation of the donor liver, the portal vein and the caval vein of the donor organ were connected by end-to-end anastomosis to the portal vein and the caval vein of the recipient, respectively, followed by connection of the common biliary duct of the donor organ to the intestine of the recipient (Cuff technique; 20G catheter). Hindlimb ischemia was induced one to two weeks after organ transplantation.

**Isolation of murine liver progenitor cells.** Murine livers from C57BL6/N mice were dissected, liver cell suspensions prepared by mechanical dissociation. For Fluorescence Activated Cell Sorting (FACS), murine livers from C57BL6/N mice or in some experiments from GFP tg C57BL6/J mice were incubated with Collagenase Type IV (Sigma, St. Louis, Montana) for one hour under continuous shaking at 37°C. Then, cells were stained with PE labeled CD45 antibodies and APC-labeled c-kit antibodies (both ebiosciences) and sorted by using a modular MoFlo device and Summit software (Cell Sorting Facility of the Institute of Molecular Immunology at the Helmholtz Center for Environment and Health, Munich, Germany [J.W.E.]). A total of 10^5 cells was intravenously injected into each NMRI nude mouse (Charles River, Germany) 24 h after induction of hindlimb ischemia. In addition, single cells were seeded in methylecellulose plates (Methocult GF H3434, CellSystems) with 100 ng/ml human recombinant VEGF. On day 7, colony formation was studied by differential interference contrast microscopy.

**Hindlimb ischemia model.** The proximal portion of the right femoral artery including the superficial and the deep branch as well as the distal portion of the saphenous artery were occluded. The overlying skin was closed using surgical staples. After 14 days, relative blood flow was determined by laser Doppler analysis (O2C; Lea Medizin Technik, Giessen, Germany). Prior to analysis, animals were
placed on a heating pad at 37°C to minimize variations in temperature. Calculated perfusion is expressed as the ratio of ischemic to non-ischemic hindlimb perfusion.

**Tracking of mobilized progenitor cells in mice.** In order to more precisely define the phenotype of mobilized tissue resident progenitor cells, we characterized organ-derived cells in the peripheral blood by flow cytometry. In the allogeneic rat liver transplantation model, liver-derived progenitors were identified by expression of DA rat specific MHC class I molecules (APC-labeled mouse anti-rat RT1Aa Class I monoclonal antibody, Clone MN4-91-6; GeneTex; San Antonio; Texas), lack of CD45 expression (PE-labeled antibody; Pharmingen) and positive staining for c-kit (FITC-labeled antibodies; Santa Cruz Biotech, Santa Cruz, California). In the reverse GFP bone marrow transplantation model, non-bone marrow-derived progenitors were identified by GFP expression, lack of CD45 expression and positive staining for c-kit (both from ebioscience). The endothelial phenotype of the cells was determined by staining for CD45, c-kit, and CD146 (mature endothelial cells marker; all from ebioscience).

**Tracking of incorporated LacZ or GFP transgenic cells in mice.** For the identification of LacZ cells, sections were permeabilized in 0.3% Triton X-100 (Roche) and double-stained with anti-β-galactosidase monoclonal antibodies (labeled with Alexa555; Sigma) and antibodies against the endothelium-associated antigens CD31 (FITC-labeled antibodies; ebiosciences). GFP cells were identified by double staining for GFP (FITC-labeled antibody; Invitrogen; Karlsruhe; Germany) and CD31 (APC-labeled antibody; ebioscience). Nuclei were identified by Topro (for LacZ staining) or Dapi (for GFP staining). TPC frequency was defined as the number of vessels containing transgenic endothelial cells divided by the total vessels examined in representative sections. Non-ischemic skeletal muscles from wild type animals served as negative controls and non-ischemic tissue from transgenic animals served as positive controls. All images were generated on a Zeiss LSM 510 Confocal Laser Scanning Microscope.

**Tracking of incorporated male cells.** To identify cells that were derived from the transplanted male organs, we applied combined immunostaining for the endothelial marker vWF labeled with FITC (Acris;
Hiddenhausen; Germany) and interphase FISH using the rat Y-chromosome probe 9.1ES8 (a generous gift of Barbara Hoebee; Laboratory of Toxicology, Pathology, and Genetics, National Institute of Public Health and the Environment, Bilthoven, The Netherlands) according to a slightly modified FICTION protocol published by Martin-Subero and colleagues 9. Briefly, for FICTION, the cryosections were thawed and dried for 30 minutes at room temperature, fixed in acetone for 10 minutes, and air-dried. Slides were incubated for 30 minutes at room temperature with the FITC-labeled vWF antibody (1:100) diluted in PNM buffer (5% no-fat milk/PN buffer [0.1 M NaPO4 buffer pH 8.0]). After immunophenotyping, slides were fixed in Carnoy’s fixative (ethanol:acetic acid, 3:1) for 10 minutes and in paraformaldehyde solution (1%) for 1 minute. Then, slides were dehydrated through increasing ethanol concentrations and air-dried. DNA extraction, labeling with Spectrum Orange (Vysis, Downers Grove; Illinois), and preparation of the Y-chromosomal FISH probe were performed as previously described 9. On each section, 1.5 µL of the hybridization solution was applied and both probe and target DNA were simultaneously denatured at 70°C for 12 minutes and incubated for two days at 37°C. After hybridization, slides were washed in 0.1 x standard saline citrate at 60°C followed by a wash step with PNM buffer. Finally, nuclei were stained with DAPI (4’,6-diamidino-2-phenylindole, Roche Diagnostics, Mannheim, Germany) and the slides were mounted in anti-fade solution for analysis. Slides were evaluated using a Zeiss Axioskop 2 microscope equipped with appropriate filter sets and documented using the ISIS software package (MetaSystems, Altluhsheim, Germany). In female rats transplanted with either liver or small intestine from a male donor, at least 200 vWF-positive cells per section and at least three slides per animal were analyzed.

References


